Technical/Final Report
to
Saskatchewan Agriculture Research Fund

High Temperature Fermentation of Straw

10-84-07
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EXECUTIVE SUMMARY

SARF Grant #10-84-07
High Temperature Fermentation of Straw

D. E. Caldwell.
Dept. Applied Microbiology and Food Science. University of Saskatchewan.
Saskatoon, Saskatchewan S7N 0W0.

Extremely High Temperature Saccharification and Fermentation of Agricultural Residues.

D. E. Caldwell. Dept. Applied Microbiology and Food Science. University of Saskatchewan. Saskatoon, Saskatchewan S7N 0W0.

The objective of research was to determine the value of extreme thermophiles in the fermentation and saccharification of cellulose. Lactic acid was the primary product of fermentation and the strains obtained may be useful in accelerating processes for silage and/or yogurt production. The potential advantages of applying extremely thermophilic organisms include the reduction of cellulase activation energy at higher temperatures, reduced microbial contamination, thermal self-sufficiency, continuous distillation of volatile fermentation end products, and elimination of pretreatment steps.

A total of 73 thermophiles were isolated from steam-heated, cellulose-amended soil enrichments. These strains were preserved by freezing at -20 C in 50% (w/w) glycerol and cultivated using a minimal salts medium containing carboxymethyl cellulose and a small amount of yeast extract which provided unknown substances necessary for growth. The key growth substance was identified as glutamic acid. Vitamins and other nutrients had no stimulatory effect. Of the 78 strains isolated 28 were selected for study. The effect of temperature was determined for each of the isolates. Among these isolates the maximum growth temperature was 75 C, the minimum was 35 C and the optimum was generally 65 C. Several strains grew at a specific growth rate of 3.0 per hour which is equivalent to a cell doubling time of approximately 15 minutes. This is more rapid than the growth rate of most other comparable mesophilic and thermophilic organisms. The following fermentation end products were assayed:

ethanol, acetone, acetate, butanol, butyrate, crotonate, and lactate. The sole fermentation end product deteced was lactic acid. No carbon dioxide was produced, thus indicating homofermentation. Approximately 1 g per liter lactic acid was obtained.

FINAL REPORT

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High Temperature Fermentation of Straw

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Abstract

A total of 73 thermophiles were isolated from steam-heated, cellulose-amended soil enrichments. These strains were preserved by freezing at -20 C in 50% (w/w) glycerol and cultivated using a minimal salts medium containing carboxymethyl cellulose and a trace of yeast extract. The key growth substance supplied by yeast extract was identified as glutamic acid. Vitamins and other nutrients had no stimulatory effect. Of the 78 strains isolated 28 were selected for study. The effect of temperature was determined for each of the isolates. Among these isolates the maximum growth temperature was 75 C, the minimum was 35 C and the optimum was generally 65 C. Several strains grew at a specific growth rate of 3.0 per hour which is equivalent to a cell doubling time of approximately 15 minutes. This is more rapid than the growth rate of most other comparable mesophilic and thermophilic organisms. The following fermentation end products were assayed: ethanol, acetone, acetate, butanol, butyrate, crotonate, and lactate. The sole fermentation end product detected was lactic acid. No carbon dioxide was produced, thus indicating homofermentation. Approximately 1 g per liter lactic acid was obtained.

Background and Objectives

The objective of research was to determine the value of extreme thermophiles in the fermentation and saccharification of cellulose. Lactic acid was the primary product of fermentation and the strains obtained may be useful in accelerating processes for silage and/or yogurt production. The potential advantages of applying extremely thermophilic organisms include the reduction of cellulase activation energy at higher temperatures, reduced microbial contamination, thermal self-sufficiency, continuous distillation of volatile fermentation end products, and elimination of pretreatment steps.

Methods and Results:

A total of 73 thermophiles were isolated from steam-heated, cellulose-amended soil. These strains were preserved by freezing at -20 C in 50% (w/w) glycerol. The isolates were cultivated using a minimal salts medium containing carboxymethyl cellulose and a small amount of yeast extract. The yeast extract provided unknown substances necessary for growth. In subsequent experiments using synthetic media, the missing growth substance was identified as glutamic acid. Vitamins and other nutrients had no effect.

Of the strains isolated, 28 were selected for detailed study. These were 840412-G, 840412-H, 840412-K, 840412L, 840412-J, 840412-M, 840518-A, 840518-B, 840518-C, 840518-D, 840518-E, 840518-F, 840518-J, 840526-A, 840526-B, 840526-C, 840526-D, 840526-E, 840526-F, 840526-G, 840526-J, 840526-K, 840526-L, 840526-M, 840526-N, 840614-A, 840614-C, and 840711-C. The effect of temperature on each of the isolates was determined. This was necessary for subsequent studies of fermentation and cellulose degradation. Figures 1-3 show the thermal properties for 6 of the 28 strains. Many strains grew at a specific growth rate of 3.0 per hour which is equivalent to a cell doubling time of approximately 15 minutes. This is more rapid than the growth rate of comparable mesophilic organisms. From each plot the cardinal temperatures were determined.

Each of the 28 strains was tested for substrate utilization at its optimum temperature. A total of 12 substrates were tested (glu, fru, gal, man, ara, xyl, rha, suc, lac, cel, mal, ino) Results are shown in Table 1.

The following fermentation end products were assayed: ethanol, acetone, acetate, butanol, butyrate, crotonate, and lactate. Ethanol was determined both by gas chromatography and

enzymatically. Alcohol dehydrogenase was used to covert ethanol and NAD to acetaldehyde and NADH. The NADH was measured spectrophotometrically at 340 nm. Lactic acid and NAD were converted to NADH and pyruvate using lactic dehydrogenase. The NADH formed was measured spectrophotometrically at 340 nm. The remaining end products were determined by gas chromatography. The sole fermentation end product was lactic acid. No carbon dioxide was produced and this indicated a homofermentation. Approximately 1 g per liter of lactic acid was produced.

Discussion and Impact:

Lactic acid was the primary product of fermentation rather than ethanol. However, traces of ethanol were detected and it may be possible to increase ethanol production by changing the conditions of fermentation, the redox state of the substrates, or through genetic engineering. Presently, it is not possible to simultaneously ferment cellulose and distill ethanol using these strains. However, the thermophiles described are unusual in that they grow more rapidly than similar strains isolated by other workers. Their rapid growth rate (doubling time of approximately 15 min. for several strains) indicates that they may be metabolically more effective than are analogous mesophiles. Like ethanol, lactic acid is a useful microbial product and processes for thermophilic production of yogurt or silage are potential applications which remain to be explored.

Agricultural fermentations is an area often neglected because it involves the utilization of agricultural products rather than their production. However, the creation of new uses for agricultural products is of primary importance to the success of the agricultural industry. It increases market demand for agricultural products and consequently raises prices. There is little value in increased production without a corresponding increase in demand. The characterization of new microorganisms for use in agriculture has helped to acheive this goal.

Extremely High Temperature Saccharification and Fermentation of Agricultural Residues.

D. E. Caldwell. Dept. Applied Microbiology and Food Science. University of Saskatchewan. Saskatoon, Saskatchewan S7N 0W0.

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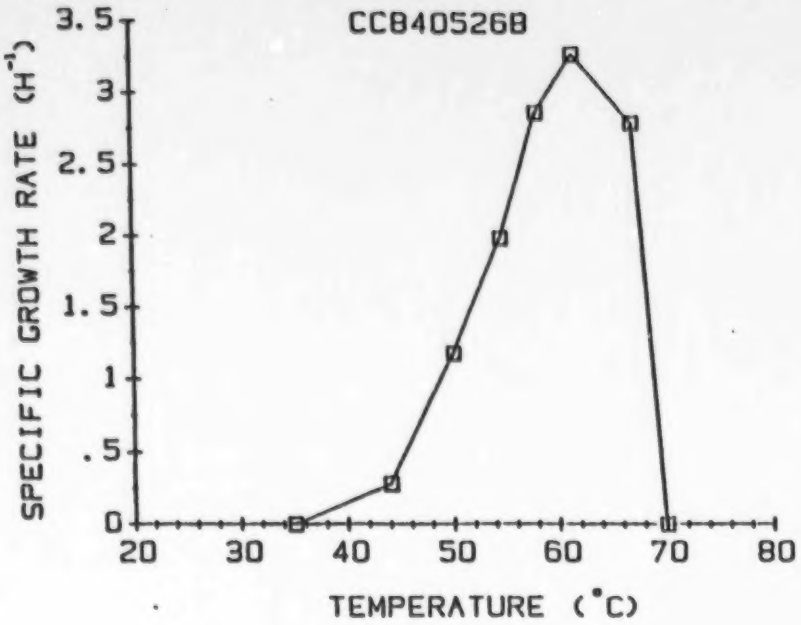
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70
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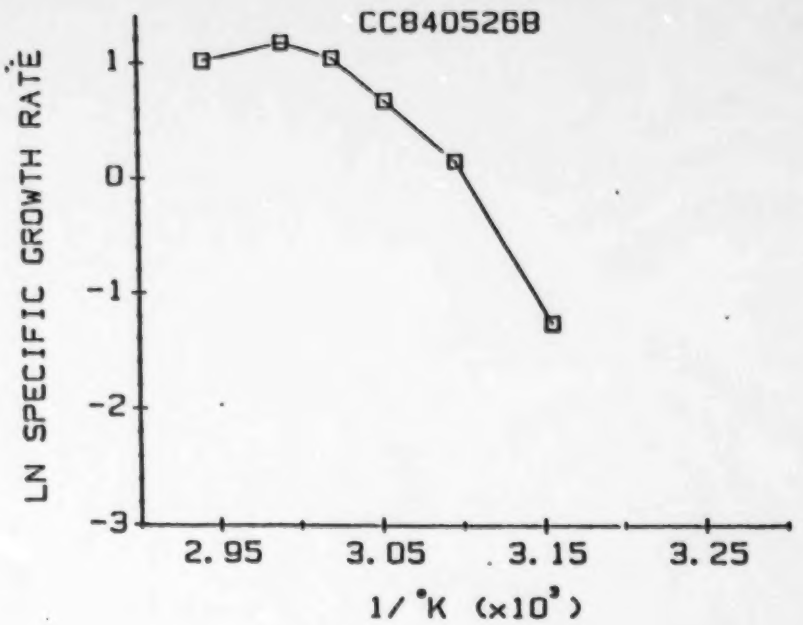
TEMPERATURE (°C)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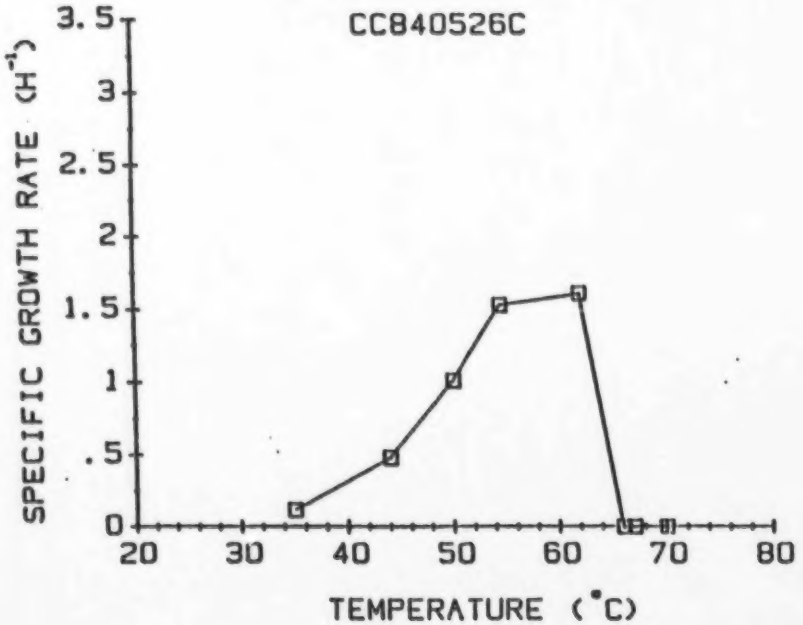
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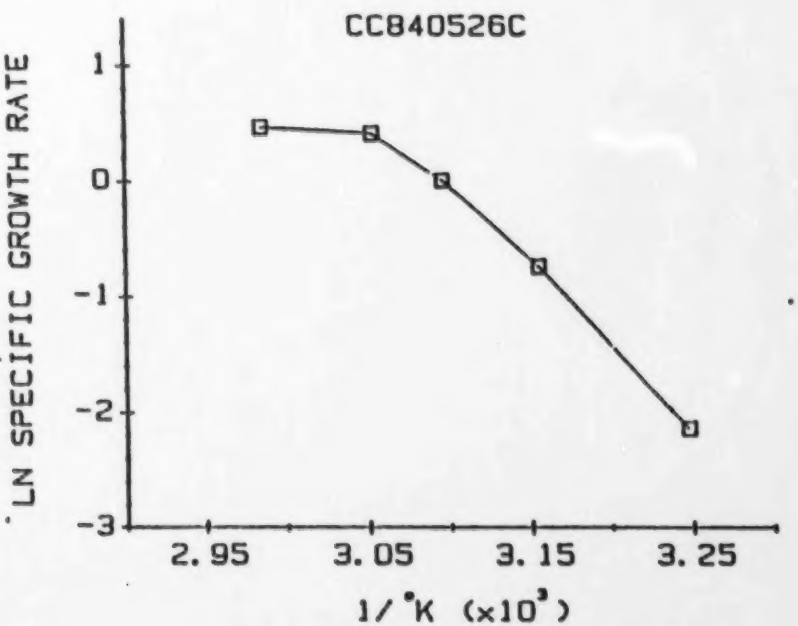
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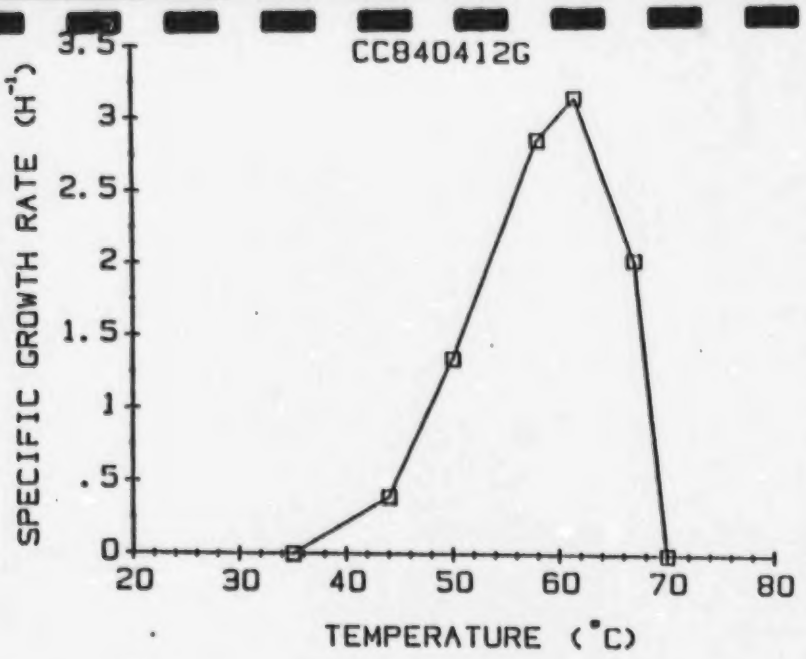
FIGURE 1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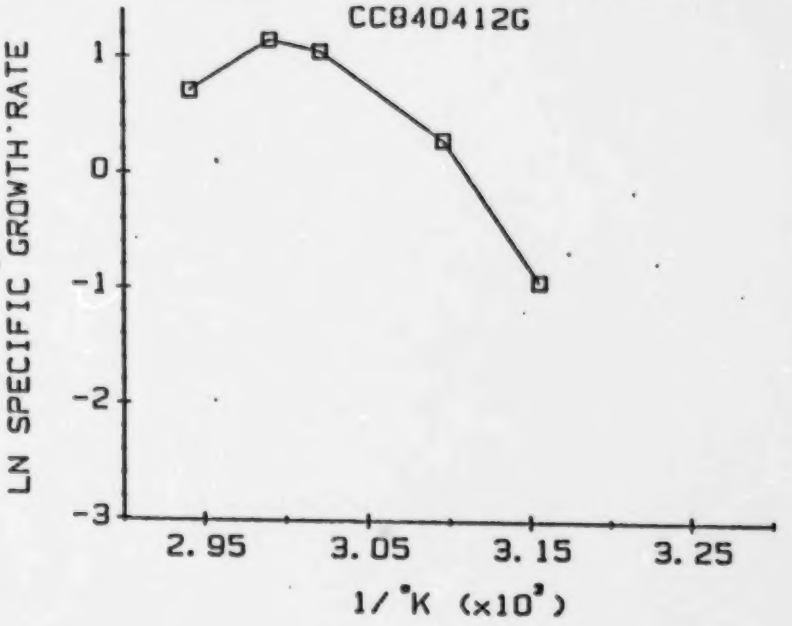
TEMPERATURE (°C)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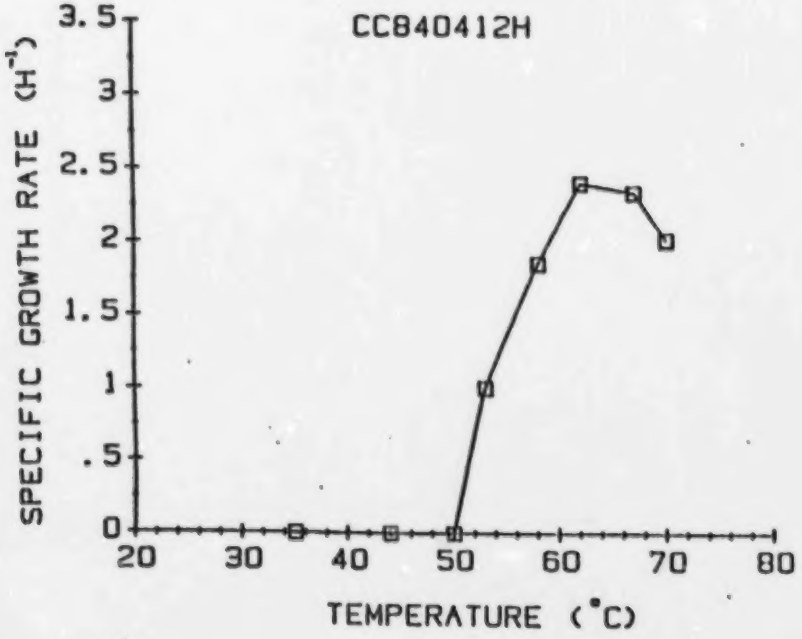
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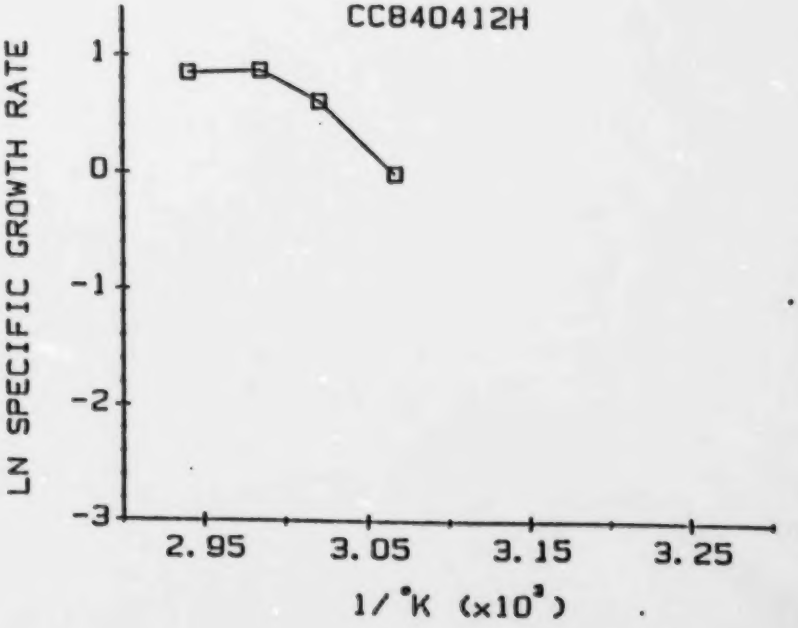
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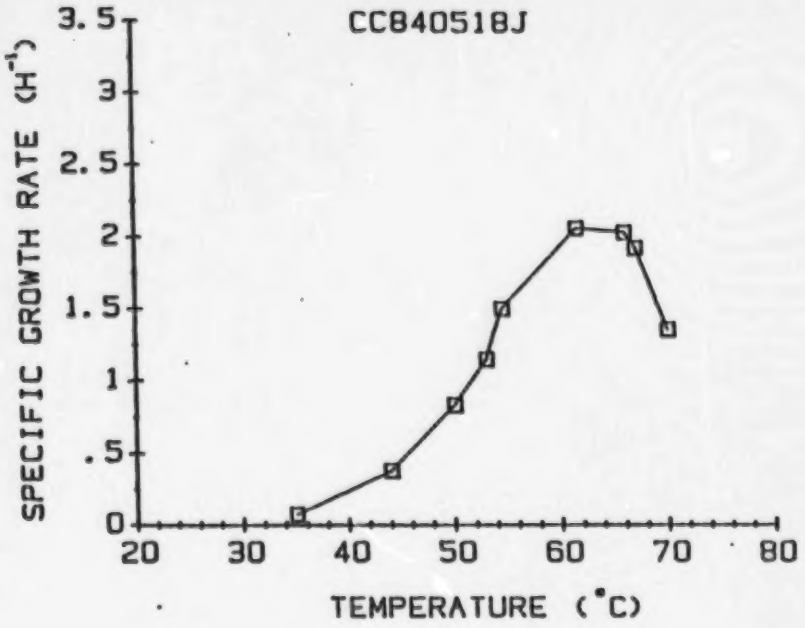
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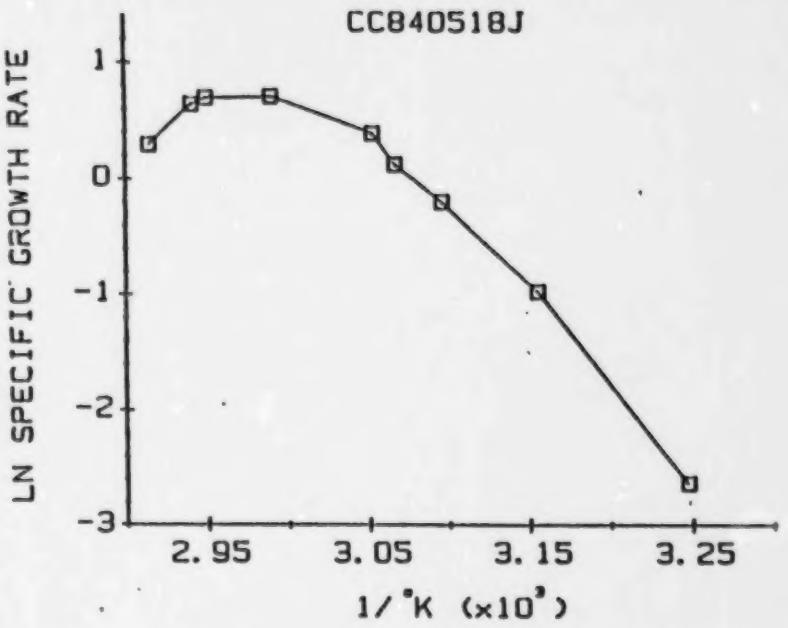
TEMPERATURE (°C)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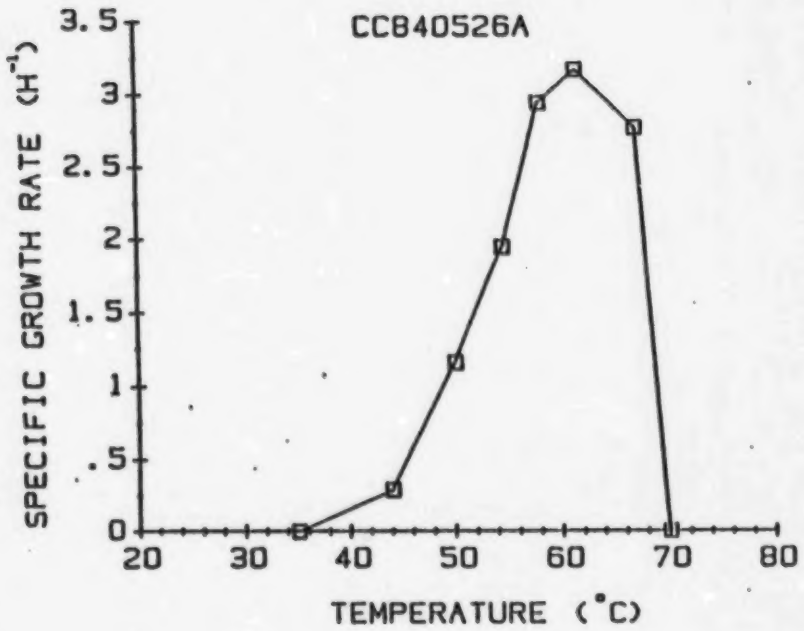
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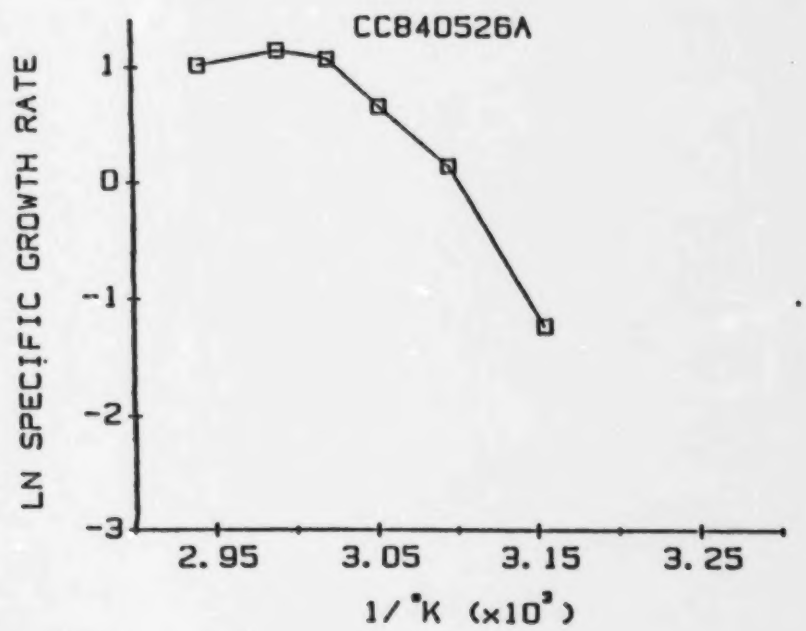
TEMPERATURE (°C)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1/°K (x10³)

ISOLATE	Glu	Fru	Gal	Man	Ara	Xyl	Rha	Suc	Lac	Cel	Mal	Ino
CCS840412G	+	+	+	+	-	+	-	-	+	+	+	+
CCS840412H	+	+	-	+	-	+	-	+	+	+	+	+
CCS840412J	+	-	+	+	+	+	-	+	+	-	+	-
CCS840412K	+	+	-	+	-	+	-	+	-	+	+	+
CCS840412L	-	+	+	+	-	+	-	+	+	+	+	+
CCS840412M	+	+	-	+	+	+	+	+	+	+	+	+
CCS840518A	+	+	+	+	+	+	-	+	-	-	+	-
CCS840518B	+	+	-	+	+	-	+	+	+	+	+	+
CCS840518C	+	+	+	+	-	+	-	+	-	-	+	+
CCS840518D	+	+	+	+	-	+	-	+	+	+	+	-
CCS840518E	+	+	+	+	-	+	-	+	-	+	+	-
CCS840518F	+	+	+	+	-	+	-	+	+	+	+	-
CCS840518J	+	+	+	+	+	+	+	+	+	+	+	+
CCS840526A	+	+	+	+	+	+	+	-	-	+	+	+
CCS840526B	+	+	+	+	+	+	+	+	-	+	+	-
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CCS840526K	+	+	+	+	+	+	-	+	+	+	+	-
CCS840526L	+	+	+	+	-	+	-	+	+	+	+	+
CCS840526M	+	+	+	+	+	+	-	+	+	+	+	-
CCS840526N	+	+	+	+	+	+	-	+	-	+	+	+
CCS840614A	+	+	+	+	+	+	-	-	-	+	+	-
CCS840614C	+	+	+	+	+	+	+	+	+	+	+	+
CCS840711C	+	+	+	+	-	+	-	+	+	-	+	+

EXECUTIVE SUMMARY

SARF GRANT #10-83-04

HIGH TEMPERATURE FERMENTATION OF STRAW

April 1, 1984 to March 31, 1985

Douglas E. Caldwell

Thermophilic bacteria have been isolated to improve the economics of fermentation and create new markets for agricultural residues, such as flax straw, which cannot be returned to the soil or used as feed. Successful completion of the objectives will provide a new, more cost effective, means of converting agricultural residues and non-crop plants to sugar and/or fermentation end products such as ethanol (gasoline additive) and butanol (diesel fuel additive). The process could potentially be carried out on individual farms, in cooperative processing facilities, or industrially. The lignin portion of the feedstock will be retained and added to soil. This will conserve soil organic matter (humus) while

harvesting the energy present in cellulose (which is otherwise lost when agricultural residues are returned directly to soil and directly decomposed without microbial processing).

Thermophilic bacterial isolates were obtained from in situ soil enrichments consisting of continuously steamed soil amended with carboxymethyl cellulose at daily intervals for a period of one month. The resulting isolates were characterized and are being evaluated for the ability to hydrolyze and ferment cellulose. Results indicate that the thermal optima of the isolates are below the 78 C optimum for distillation of ethanol. The potential advantages of applying extremely thermophilic organisms include the reduction of cellulase activation energy at higher temperatures, reduced microbial contamination, thermal self-sufficiency, continuous distillation of volatile fermentation end products, and elimination of pretreatment steps. Due to the high maintenance rate of extreme thermophiles, the isolated enzyme will be more effective than cell cultures in achieving efficient saccharification. Thus the economic feasibility of using the isolated cellulase enzyme system, as opposed to cell cultures, may be determined in future studies.

TECHNICAL REPORT

SARF GRANT #10-83-04

HIGH TEMPERATURE FERMENTATION OF STRAW

APRIL 1, 1984 TO MARCH 31, 1985

DOUGLAS E. CALDWELL

Abstract

Thermophilic bacterial isolates were obtained from in situ soil enrichments consisting of continuously steamed soil amended with carboxymethyl cellulose at daily intervals for a period of one month. The resulting isolates were characterized and are being evaluated for the ability to hydrolyze and ferment cellulose. Results indicate that the thermal optima of the isolates are below the 78 C optimum for distillation of ethanol. The potential advantages of applying extremely thermophilic organisms include the reduction of cellulase activation energy at higher temperatures, reduced microbial contamination, thermal self-sufficiency, continuous distillation of volatile fermentation end products, and elimination of pretreatment steps. Due to the high maintenance rate of extreme thermophiles, the isolated enzyme will be more effective than cell cultures in achieving efficient saccharification. Thus the economic feasibility of using the isolated cellulase enzyme system, as opposed to cell cultures, may be determined in future studies. If use of the enzyme should prove cost effective, a catabolite repression negative mutant would be of value.

Background

There are numerous advantages in using extremely thermophilic organisms for the hydrolysis of cellulosic agricultural by-products. These include the reduction of cellulase activation energy at higher tempertures, reduced microbial contamination, thermal self-sufficiency, and continuous distillation of volatile fermentation end products. Reductions in cellulose activation energy result in more rapid hydrolysis. However, there are no Arrhenius plots for either the organisms or their cellulase enzyme systems between 70 and 80 C. Since extreme thermophiles are not commonly found in ambient enviroments, the chances of microbial contamination during industrial use is minimal. large scale fermentation systems require expensive cooling systems to remove the heat produced during microbial metabolism. However, the effect of self heating is beneficial in the case of themophilic orgnaisms. In addition, volatile end products can be continuously distilled at high temperature. This saves the expense of the first distillation step in fuel alcohol production. It also prevents inhibition in the fermentor.

At present *Trichoderma spp.* (fungi), *Cellulomonas spp.* (bacteria), and *Clostridium thermocellum* (bacteria) are most commonly used in studies of bacterial cellulose

hydrolysis. None of these organisms is considered to be part of the "new biotechnology". *Clostridium thermocellum*, originally isolated in 1926 (13), ferments cellulose optimally at between 60 and 65 C at approximately the same rate as *Trichoderma spp.*. *Cellulomonas* spp. generally ferment at a slower rate.

During the last five years numerous extreme thermophiles have been discovered which prefer temperature from 70 to well above 100 C. These new "extreme" thermophiles have yet to be applied to industrial processes although they are considered to be an important aspect of the "new biotechnology". Organisms growing above 100 C present new problems since industrial fermentation systems have not yet been designed to function in this temperature range and laboratory systems have been in use only recently (since 1983). However, cellulolytic orgnaisms capbale of growing between 70 and 100 C have immediate industrial potential because they can be cultivated using traditional fermentors.

Approximately 50% of the cellulose in straw exists in a crystalline form which is resistant to microbial attack. From the lumen to the middle lamella of the cell wall the concentration of lignin varies from a concentration of nearly zero to more than 95%. The combination of high crystallinity and the presence of the relatively nono-biodegradable lignin polymer makes native cellulose very difficuylt to degrade enzymatically or microbiologically. As a result it is

generally necessary to pretreat cellulose to achieve effective degradation. However, using extremely thermophilic microbial technology may eliminate the need for a separate pretreatment since the temperature of incubation (75 C) may serve as a substitute for pretreatment.

Objectives

1. Bacteriological and biochemical description of isolates.

2. Optimization of saccharification (conditions for glucose production).

3. Determination of cellulose hydrolysis rates and products.

Experimental Method

Organisms were cultivated using the following improved cellulose isolation medium (ICIM) which incorportates glutamic acid for yeast extract (concentrations are given in grams per liter).

Carboxymethyl cellulose (5), l-glutamic acid (0.2), ferric ammonium citrate (0.2), sodium chloride (2), ammonium chloride (1), magnesium sulfate (1), KA 940 Gellan Gum

(thermostable gelling agent)(10), potassium phosphate (2.7), sodium phosphate (4.24), 5 ml trace element solution.

The trace element solution consisted of: EDTA (10), calcium chloride (3) iron sulfate (0.2), manganese sulfate (0.1), sodium molybdate (0.1)

The phosphate solution and trace elements were autoclaved separately to prevent precipitation. An autoclave time of 90 minutes was used to ensure killing of heat-stable thermophilic spores.

Results and Discussion

Thus far a total of 73 isolates have been obtained. This includes aerobes and anerobes. These organisms have been preserved at -20 C in a 50% (w/w) glycerol solution. They were cultivated using a minimal salts medium containing yeast extract and carboxymethyl cellulose. The yeast extract provided unknown substances ncecessary for growth and glutamic acid has been identified as the factor required. This information has been used to formulate a synthetic medium ans is an important step in microbial characterization. It has also been determined that although many of the isolates are capable of growth at 78 C (the distillation temperature of ethanol) the optimal temperature is below 78. Further studies are being conducted to

determine the batch growth curve, maximum specific growth rate, and biochemical characteristics of the isolates. Once they have been characterized and the optimal growth conditions determined, the hydrolysis products and rates of cellulose degradation will be determined.

Impact

Successful completion of the objectives will provide a new and more cost-effective means of converting agricultural residues and non-crop plants to sugar and/or fermentation end products such as ethanol (gasoline additive) and butanol (diesel fuel additive). The process could potentially be carried out on an individual farm, in a cooperative processiing facility, or industrially.

No bacteria have yet been isolated which are capable of fermenting cellulose at the distillation temperature for ethanol (78 C). Such organisms could substantially improve the economics of fermentation and create new markets for agricultural residues, such as flax straw, which cannot be returned to the soil or used as feed.

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